6R型テトラヒドロビオプテリンの海馬におけるアセチルコリン遊離促進作用 - 脳微小透析法による特徴化(Dissertation_全文)

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主論文 1

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Monoamine-mediated enhancement of acetylcholine release in rat hippocampus by 6*R*-L-erythro-5,6,7,8-tetrahydrobiopterin

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Enhancement of acetylcholine release in the hippocampus by 6R-L-erythro-5,6,7,8-tetrahydrobiopterin is mediated by 5-hydroxytryptamine

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Abbreviations used: 6R-BH_4, 6R-L-erythro-5,6,7,8-Tetrahydrobiopterin; α-MT, α-Methyl-p-tyrosine; PCPA, p-Chlorophenylalanine; 5-HT, 5-Hydroxytryptamine; L-DOPA, L-3,4-Dihydroxyphenylalanine

Key Words: Acetylcholine release; Biopterin; Hippocampus; rain microdialysis; p-Chlorophenylalnine; 5-HT; α-Methyl-p-tyrosine; Catecholamine

Recently, we reported that 6R-L-erythro-tetrahydrobiopterin (6R-BH_4), a natural cofactor for L-aromatic amino acid hydroxylases, enhances in vivo release of acetylcholine (ACh) in the rat hippocampus: the enhancement was abolished after depletion of brain catecholamines and 5-hydroxytryptamine (5-HT) by pretreatment with reserpine. In the present study, we have used in vivo brain microdialysis to clarify the neuronal mechanism involved in the enhancement of ACh release by 6R-BH_4. After depletion of catecholamines by pretreatment of rats with α-methyl-p-tyrosine, 6R-BH_4 added to the perfusion fluid still induced an increase in extracellular ACh levels monitored by microdialysis as an index of ACh release. In contrast, after depletion of 5-HT by pretreatment with p-chlorophenylalanine, most of the 6R-BH_4-induced enhancement was eliminated. Exogenous 5-HT and dopamine (DA) but not noradrenaline added to the perfusion fluid stimulated ACh release with 5-HT being far more potent. Intraperitoneal administration of 5-hydroxytryptophan and L-DOPA also enhanced ACh release, presumably by their conversion to 5-HT and catecholamines, respectively. Administration of 6R-BH_4 increased hippocampal 5-HT release, as indicated by increased extracellular levels of the major 5-HT metabolite, 5-hydroxyindoleacetic acid. These results suggest that 6R-BH_4 stimulates ACh release in the hippocampus, mainly by augmenting release of 5-HT, a potent stimulator of ACh release, and partly by augmenting release of DA.

INTRODUCTION

6R-L-erythro-5,6,7,8-Tetrahydrobiopterin (6R-BH_4) is a natural cofactor for phenylalanine[6], tyrosine[21] and tryptophan[16] hydroxylases. The distribution of 6R-BH_4 in the brain is positively correlated with the activities of tyrosine and tryptophan hydroxylases[1,14,15]. In addition, these hydroxylases seem to be unsaturated with tissue levels of 6R-BH_4, since its administration enhances the synthesis of brain catecholamines[10,18] and 5-hydroxytryptamine (5-HT)[18,27]. Thus, 6R-BH_4 has been considered to play an important role in regulation of neurotransmitter monoamine biosynthesis.

In our recent reports using in vivo brain microdialysis, administration of 6R-BH_4 was found to enhance in vivo release of dopamine (DA)[11,17] and 5-HT[17] in the striatum of rats; it also enhanced the release of acetylcholine (ACh) in the hippocampus[23,24], and of glutamate in the striatum and frontal cortex[17]. Furthermore, the enhanced release of glutamate in the striatum by 6R-BH_4 was shown to be mediated by the DAergic neuronal system, since it was abolished after destruction of striatal DAergic nerve terminals by intrastriatal injection of 6-hydroxydopamine[17]. Similarly, enhancement of ACh release in the hippocampus seems to be mediated by monoaminergic neuronal systems, since it was abolished after depletion of both catecholamines and 5-HT by reserpine[23].

However, since reserpine is known to deplete both catechol-

amines and 5-HT[30], it is presently unknown which of these monoaminergic systems (catecholaminergic, 5-HTergic or both) is involved in the enhancement of ACh release by 6R-BH_4. It is also unknown whether the involved monoaminergic system is activated or depressed by 6R-BH_4 and what effects (stimulatory or inhibitory) on ACh release the monoamine neurotransmitters exert. To clarify these points, the effects of 6R-BH_4 on ACh release were examined following selective depletion of catecholamines or 5-HT. The effects of monoamines themselves and their precursors, such as 5-hydroxytryptophan (5-HTP) and L-DOPA administration on ACh release were also examined. Finally, the effects of 6R-BH_4 on release of monoamines were examined.

MATERIALS AND METHODS

Brain microdialysis

Male Wistar rats weighing 250-300 g were lightly anesthetized with diethyl ether and mounted on a stereotaxic apparatus (incisor bar 3.3 mm below the auricular horizontal plane). The skull was exposed by a midline skin incision and a burr hole of 3 mm diameter was drilled. A U-shaped probe for microdialysis (BDP 23-03, EICOM, Kyoto, Japan) was stereotactically implanted into the right hippocampus (coordinates taken from the bregma with the skull flat: A, -5.8; L, 5.0; V, -6.8 mm, from the Atlas of Paxinos and Watson[25]) and fixed in place with cranioplastic cement. After the rats had recovered from the anesthesia, brain microdialysis was carried out under freely-moving conditions[11,12,22-24]. The dialysis probe was continuously perfused at a flow rate of 5.4 μl/min with Ringer solution (147 mM NaCl, 2.3 mM $CaCl_2$ and 2.3 mM KCl; pH 6.1) containing 30 ¥M physostigmine sulfate, an inhibitor of acetylcholinesterase, and the dialysates were collected every 20 min. Unless otherwise specified, dialysates were directly injected onto HPLC for analysis of ACh without purification.

Immediately before use, 6R-BH_4 dihydrochloride, noradrenaline (NA) bitartrate, DA hydrochloride or 5-HT creatinine sulfate was dissolved in the perfusion fluid to the desired concentration: the final pH of the solution was adjusted to that of Ringer solution (6.1) with 20 mM NaOH.

Purification of ACh using Sephadex G-10 columns

In experiments, where 6R-BH_4 was added to the perfusion fluid, ACh recovered in dialysates was analyzed after purification on small columns of Sephadex G-10 (Pharmacia, Uppsala, Sweden), as described recently[23]. In brief, total amount of the hippocampal dialysate was applied onto the Sephadex G-10 column (bed volume, 7 x 60 mm), which had been equilibrated with 10 mM HCOOH. After the column was washed with 1.0 ml of 10 mM HCOOH, the next 1-ml fraction of 10 mM HCOOH was collected, lyophilized to dryness, and reconstituted in 120 μl of distilled water. An aliquot (75 μl) of the solution was injected onto HPLC for analysis of ACh.

Determination of ACh in dialysates

ACh in crude dialysates or in eluates from Sephadex G-10 columns was determined using HPLC with an immobilized enzyme reactor and an electrochemical detector as described recently[8,12,22-24]. The HPLC system consisted of a Hitachi (Tokyo, Japan) L-6000 pump, a Hitachi 655A-40 automated sample injector, a separation column of polystyrene resin (Eicompak AC-Gel, 6 x 300 mm; EICOM, Kyoto, Japan), an enzyme reactor (containing acetylcholinesterase and choline oxidase) and an electrochemical detector (Eicom Model 100) with a working electrode of platinum. The potential of the electrochemical detector was set at +450 mV. The mobile phase was 100 mM sodium phosphate buffer (pH 8.0)

containing 300 mg/L sodium 1-decanesulfonate and 90 mg/L tetramethylammonium chloride, and was pumped at a flow rate of 1.0 ml/min. Quantification of ACh and choline was performed by comparison with peak heights of known amounts of authentic standards.

Purification and analysis of ACh in dialysates using a column-switching HPLC system

In experiments, where NA, DA and 5-HT were directly added to the perfusion fluid, ACh in dialysates was purified and analyzed using a column-switching HPLC method, as described recently[22]. In this system, a short column of polystyrene (called "isolation column" vs. separation column) and the second pump were added to the above-mentioned standard HPLC system for ACh analysis, to remove these monoamines from the samples. The isolation column was located on the upstream of the separation column through a switching valve.

For analysis, the dialysate sample containing monoamines was directly injected onto the HPLC system. When choline and ACh in the sample had been eluted from the isolation column but monoamines were still retained on the column, the position of the switching valve was changed so that the isolation column was isolated from the main route. After this switching, the isolation column was washed with the mobile phase driven by the second pump through the switching valve, to elute the monoamines still retained on the column out of the system: the main column

continued to be perfused with the mobile phase from the main pump. As a result, the earlier eluate from the isolation column, which contained ACh and choline but not monoamines, was advanced to the main column for a subsequent analysis. After both analysis of choline and ACh and washing of the isolation column had been completed, the switching valve was restored to the initial position and the next injection of the dialysate was performed.

The mobile phase was the same as that for the standard ACh analysis system, which was delivered at flow rates of 1.0 ml/min and 2.0 ml/min with the main pump and the subsidiary pump, respectively.

Determination of monoamines in dialysates and in tissue extracts

In some experiments, the contents of NA, DA and 5-HT in homogenates of the hippocampus and the rest of the whole brain was simultaneously determined, using HPLC with electrochemical detection[13,18-20,23,24,29]. That is, the hippocampus and the rest of the whole brain were homogenized in 1 ml and 10 ml, respectively, of 0.1 M perchloric acid containing 1 mg/ml sodium bisulfite and 1 mM EDTA, and the homogenate was centrifuged at 17,500 x g for 15 min at 4°C. The resulting supernatant was passed through a membrane filter (pore size, 0.45 μM), and an aliquot of the filtrate was injected onto HPLC with electrochemical detection. The mobile phase was 100 mM citrate/sodium acetate buffer (pH 3.8) containing 15% methanol, 100 μM EDTA and

150 mg/L sodium-1-octanesulfonate, which was pumped at a flow rate of 1.0 ml/min. A $5C_{18}$ reverse-phase column (Cosmosil, 4.6 x 150 mm; Nacalai Tesque, Kyoto, Japan) was used for separation. The working electrode of the electrochemical detector was of graphite carbon, and its potential was set at +700 mV against the Ag/AgCl reference electrode.

NA and DA in dialysates were analyzed after purification with a small-scale alumina batch method, using HPLC with electrochemical detection, as described elsewhere[11]. 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in dialysates were simultaneously analyzed using HPLC with electrochemical detection, as described recently[3,4].

Procedures for monoamine depletion in the brain

In some experiments, rats were treated with inhibitors of tyrosine hydroxylase (α-methyl-p-tyrosine, α-MT) or tryptophan hydroxylase (p-chlorophenylalanine, PCPA) to deplete catecholamines or 5-HT in the brain, respectively. α-MT was intraperitoneally injected three times (20 h, 4 h and 2 h) before experiments: the doses of the first, the second and the last injections were 200 mg/kg, 200 mg/kg and 100 mg/kg, respectively. PCPA was injected intraperitoneally 48 h before experiments at the dose of 400 mg/kg.

Materials

Reagents were obtained from the following sources: ACh

bromide, physostigmine sulfate, NA bitartate, DA hydrochloride 5-HT creatinine sulfate and 5-HIAA from Wako Pure Chemical Industries, Osaka, Japan; sodium 1-decanesulfonate and sodium 1-octanesulfonate from Tokyo Kasei Kogyo, Tokyo, Japan; PCPA and α-MT from Sigma, St. Louis, U.S.A. 6R-BH_4 dihydrochloride was a generous gift from Suntory Institute for Biomedical Research, Osaka, Japan.

Statistical analysis

All results were expressed as means ± S.E.M. The data were subjected to a two-way analysis of variance, and when significant F values were encountered, Neumann-Keuls' multiple-range test was used to test for significant differences between treatment means[28]. A probability level of $p < 0.05$ was considered statistically significant.

RESULTS

As described recently[23], ACh levels in dialysates increased in a concentration-dependent manner following infusion of various concentrations of 6R-BH_4 and reached the maximum in the fraction collected between 20 and 40 min: the maximal level was 6-fold of the basal level at a final concentration of 1 mM (Fig. 1A).

When 6R-BH_4 was given after pretreatment with α-MT, a selective inhibitor of tyrosine hydroxylase, it increased the ACh levels to the same extent as in the absence of the pretreatment with α-MT (Fig. 1B). In contrast, after pretreatment with PCPA, a selective inhibitor of tryptophan hydroxylase, the 6R-BH_4-induced increase in ACh levels was reduced to about 20% of the value in the absence of the pretreatment with PCPA (Fig. 1C). Both α-MT and PCPA had little effect on basal ACh levels (Figs. 1B and 1C).

Table 1 shows the effects of pretreatment with α-MT or PCPA on the contents of NA, DA and 5-HT in the hippocampus. After pretreatment with α-MT, the contents of NA and DA in the hippocampus were reduced to 5.03% and 19.8%, respectively, of the control value, but the content of 5-HT in the hippocampus was virtually unchanged. After pretreatment with PCPA, the content of 5-HT in the hippocampus was reduced to 16.7% of the control value, but the contents of NA and DA were unaffected. Essentially similar results were obtained for the rest of the brain (data not shown).

We determined the effects of exogenous monoamines added to the perfusion fluid on ACh levels in dialysates (Fig. 2). Infusion of NA up to 5 mM in the perfusion fluid was without effect on ACh levels in dialysates. In contrast, infusion of DA or 5-HT increased ACh levels in dialysates in a concentration-dependent manner: the maximal levels were approximately 200% of the basal level for DA and 400% for 5-HT.

We also examined the effects of increases in endogenous monoamines on ACh levels in dialysates (Fig. 3). For this purpose, ACh levels in dialysates were determined following administration of monoamine precursors, such as L-DOPA and 5-HTP. ACh levels in dialysates were unchanged following administration of 50 and 100 mg/kg of L-DOPA. At 200 mg/kg of L-DOPA, the ACh levels significantly increased and reached the maximum (140% of the basal level) in the fraction collected between 40 and 60 min after the injection (Fig. 3A). In contrast, the effects of administration of 5-HTP were more marked. The ACh levels increased in a dose-dependent manner following administration of various doses of 5-HTP and reached the maximum in the fraction collected between 40 and 60 min after the injection: the maximal level was 285% of the basal level at 200mg/kg of 5-HTP (Fig. 3B).

In separate experiments, we determined the changes in the monoamine content in the brain following administration of L-DOPA or 5-HTP. That is, experiments were performed according to the

same protocol as that in Fig. 3 except that the rats were killed 60 min after the administration for determination of the monoamine content in the brain. Following administration of L-DOPA, the contents of NA and DA in the hippocampus increased in a concentration-dependent manner and at 200 mg/kg, they were 134% and 285% of the control value, respectively (Table II). The content of 5-HT in the hippocampus was virtually unaffected by administration of L-DOPA. Administration of 5-HTP produced a dose-dependent increase in the content of 5-HT: the increases were 190, 280 and 540% of the control value, respectively, at the doses of 50, 100 and 200 mg/kg. Administration of 5-HTP had little effect on the contents of NA and DA in the hippocampus (Table II). Essentially similar results were obtained for the rest of the brain (data not shown).

Finally, to estimate the changes in <u>in vivo</u> release of DA and 5-HT following administration of 6R-BH_4, we attempted to determine extracellular levels of DA and 5-HT in the hippocampus. However, both DA and 5-HT in dialysates were found to be undetectable (< 50 fmol/20 min-fraction) before and after administration of 6R-BH_4. Therefore, we measured extracellular levels of DA and 5-HT metabolites as indices of release of these monoamines. DA metabolites such as DOPAC and HVA in dialysates were also undetectable (< 50 fmol/20 min-fraction) before and after administration of 6R-BH_4. In contrast, extracellular levels of 5-HIAA, a major 5-HT metabolite, were found to increase to 150 % of the basal

levels following administration of 1 mM 6R-BH_4, as shown in Fig. 4.

DISCUSSION

6R-BH_4 given through the dialysis membrane induced a marked increase in extracellular ACh levels monitored by _in vivo_ brain microdialysis, an index of ACh release (Fig. 1A), as reported recently[23].

The 6R-BH_4-induced increase in ACh release persisted after selective depletion of catecholamines by α-MT (Fig. 1B and Table I). In contrast, the major portion of the increase was eliminated after selective depletion of 5-HT by PCPA (Fig. 1C and Table I). These results suggest that the 5-HTergic system is mainly involved in the enhancement of ACh release by 6R-BH_4. However, after pretreatment with PCPA, a minor portion (20%) of the 6R-BH_4-induced increase still remained. The PCPA-resistant increase could be mediated by the 5-HT, which had remained even after pretreatment with PCPA (Table I). Alternatively, the increase might be mediated by catecholaminergic systems (DAergic and/or NAergic systems), considering the possibility that the catecholamine-mediated portion is too small to detect as a significant change (Fig. 1B).

On the other hand, the basal extracellular levels of ACh were unchanged after pretreatment with α-MT or PCPA (Fig. 1). This result indicates that there is little, if any, tonic control of catecholamines and 5-HT under basal conditions.

Exogenous 5-HT and DA (but not NA), added to the perfusion fluid, augmented ACh release with 5-HT being more potent than DA

(Fig. 2). Furthermore, an increase in release of endogenous catecholamines and 5-HT, which was expected to occur following administration of L-DOPA and 5-HTP, also enhanced ACh release with 5-HTP being more potent than L-DOPA (Fig. 3). These results taken together suggest that 5-HT and to a lesser extent, DA, which are released from monoaminergic nerve terminals, can stimulate ACh release in the hippocampus. At present, it is established that the hippocampus is innervated by the 5-HTergic and NAergic neurons as well as the cholinergic neurons[5,9], but the DAergic innervation of the hippocampus is still controversial. However, our data suggest that there is a minor DAergic projection to the cholinergic neurons in the hippocampus. Thus, combined with the results in Fig. 1, the present study strongly indicates that enhancement of ACh release by 6R-BH_4 is mediated mainly by activation of 5-HTergic system, with a minor portion being contributed to by the DAergic system.

In fact, an increase in extracellular levels of 5-HIAA, a metabolite of 5-HT, was observed following administration of 6R-BH_4 (Fig. 4). Since extracellular levels of 5-HIAA can be regarded as a good index of the rate of 5-HT release[2,7], the present observation suggests that 6R-BH_4 actually enhances 5-HT release in the hippocampus. This result is in good agreement with the previous reports that 6R-BH_4 enhances 5-HT release *in vivo* in the rat striatum as monitored by brain microdialysis[17]

and high K^+-evoked [^{3}H]5-HT release from the superfused hippocampal slice *in vitro*[31].

There, however, seems to be quantitative discrepancy. That is, administration of 6R-BH_4 to the perfusion fluid at 1 mM (which is the supramaximal concentration) markedly increased ACh levels: the increase above the control value was 500% (Fig. 1). On the other hand, the maximal increase by exogenous 5-HT or 5-HTP is 250 - 300% above the control value, and that by exogenous DA or L-DOPA is 40 - 100% above the control value (Figs. 2 and 3): the sum of these values amounts to only 290 - 400%, compared with the 500% increase for 6R-BH_4. This discrepancy might be explained by assuming that there is some synergism between the 5-HTergic and DAergic systems, like that reported for D_1 and D_2 receptors[26]. Alternatively, 6R-BH_4 alone might release 5-HT mainly into critical sites of the synapse to cholinergic neurons, which are surrounded by some diffusion barrier against exogenously administered 5-HT or 5-HTP-derived 5-HT.

In summary, we showed that 6R-BH_4 enhances ACh release in the hippocampus, mainly by augmenting release of 5-HT, a potent stimulator of ACh release, and partly by augmenting release of DA.

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LEGENDS FOR FIGURES

Fig. 1. Effects of infusion of 6R-BH_4 on extracellular ACh levels monitored by in vivo brain microdialysis in control rats and rats pretreated with α-MT or PCPA. One hundred min after the beginning of brain microdialysis, 6R-BH_4 (●) was added to the perfusion fluid at a final concentration of 1 mM during 60 min as indicated by black bars. The collected dialysate was injected onto HPLC with an elecrochemical detector after purification with a Sephadex G-10 column. (A) Control rats (pretreated with vehicle alone); (B) α-MT-treated rats; (C) PCPA-pretreated rats. Open circles represent basal ACh levels in dialysates in the absence of infusion of 6R-BH_4. Each value is the mean ± S.E.M. of 4 determinations. * $P < 0.05$; ** $P < 0.01$; significantly different from control.

Fig. 2. Effects of exogenous NA, DA and 5-HT added to the perfusion fluid on ACh levels in dialysates. One hundred minutes after the beginning of the brain microdialysis, NA, DA and 5-HT was added to the perfusion fluid at the indicated concentrations. ACh collected in dialysate was purified and analyzed using a column-switching HPLC method. An open circle (control value) represents the mean of ACh levels in five fractions of dialysates before infusion of monoamines. Closed symbols represent the

maximal ACh levels attained during infusion of the indicated concentrations of monoamines. ▲, NA; ■, DA; ●, 5-HT. Each value is the mean ± S.E.M. of 4 determinations. ** $P< 0.01$, significantly different from a control group.

Fig. 3. Effects of administration of L-DOPA and 5-HTP on ACh levels in dialysates. One hundred minutes after the beginning of the brain microdialysis, rats were injected intraperitoneally with L-DOPA (A) or 5-HTP (B) at the indicated doses. ACh levels in dialysates were monitored up to 100 min after the injection. Open bars (control value) represent the mean of ACh levels in five fractions of dialysates before administration of L-DOPA and 5-HTP. Closed bars represent the maximal ACh levels, which was attained in the third fraction (40 - 60 min) following injection of indicated doses of L-DOPA or 5-HTP. Each value is the mean ± S.E.M. of 4 determinations. ** $P< 0.01$, significantly different from a control group.

Fig. 4. Effects of infusion of 6R-BH_4 on 5-HIAA levels in dialysates. One hundred minutes after the beginning of brain microdialysis, 6R-BH_4 (●) was added to the perfusion fluid at a final concentration of 1 mM during 60 min as indicated by a black bar. The collected dialysate was directly injected onto

Table I

Effects of pretreatment with α-MT or PCPA on the contents of monoamine in the hippocampus

At the end of brain microdialysis experiments, rats were decapitated and the contents of NA, DA and 5-HT in the hippocampus were determined as described in Materials and Methods.

Each value is the mean ± S.E.M. of 4 determinations.

**, $p < 0.01$; significantly different from the corresponding control values.

	Noradrenaline (nmol/g tissue)	Dopamine (nmol/g tissue)	5-Hydroxytryptamine (nmol/g tissue)
control	3.10 ± 0.13	0.182 ± 0.006	1.26 ± 0.051
α-MT-treated	0.156 ± 0.095**	0.036 ± 0.001**	1.28 ± 0.061
PCPA-treated	3.05 ± 0.21	0.179 ± 0.003	0.211 ± 0.023**

HPLC with an elecrochemical detector for analysis of 5-HIAA. ○, control rats. Each value is the mean ± S.E.M. of 4 determinations.

** P< 0.01, significantly different from a control group.

Fig. 1

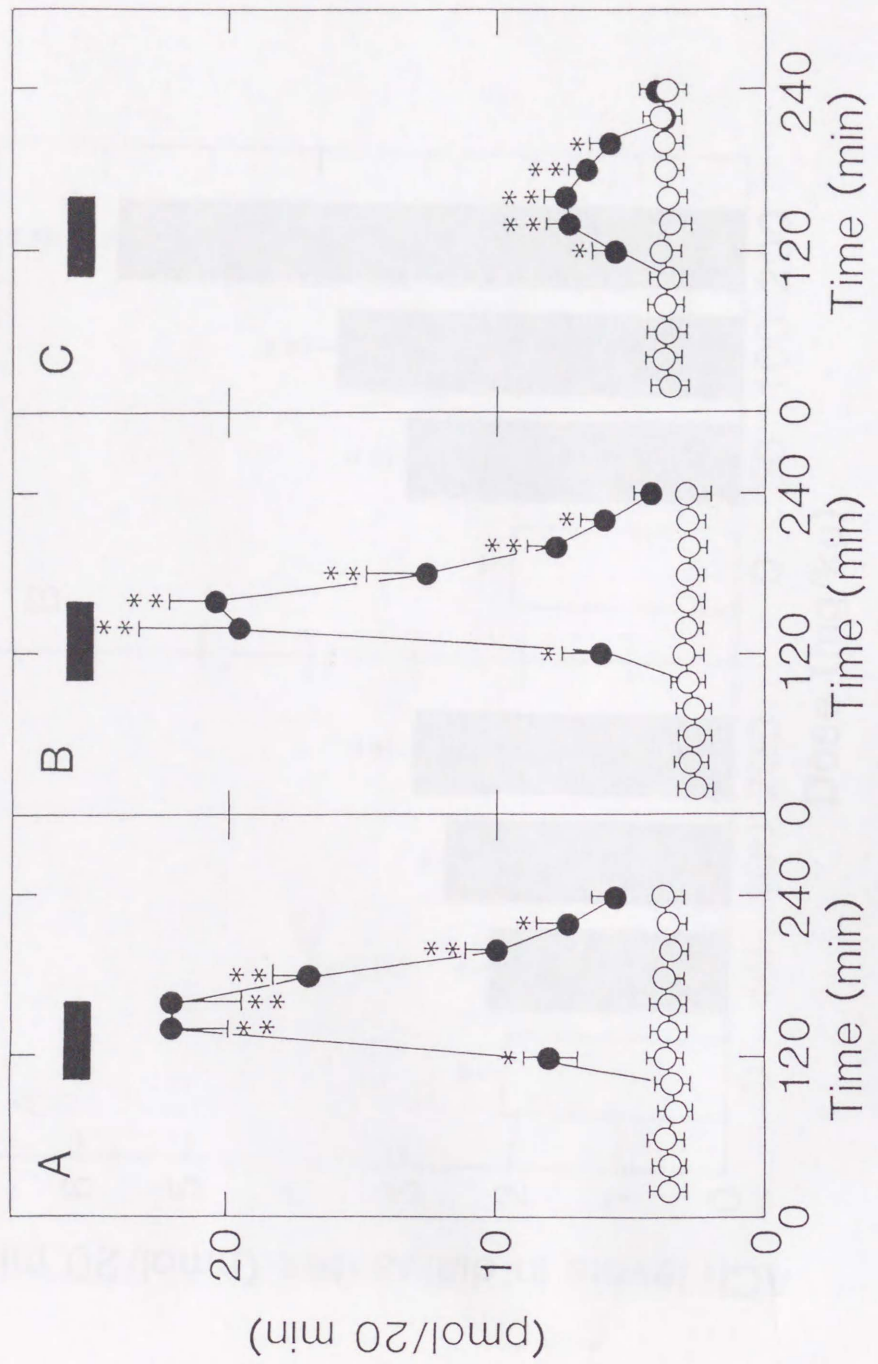


Fig. 2

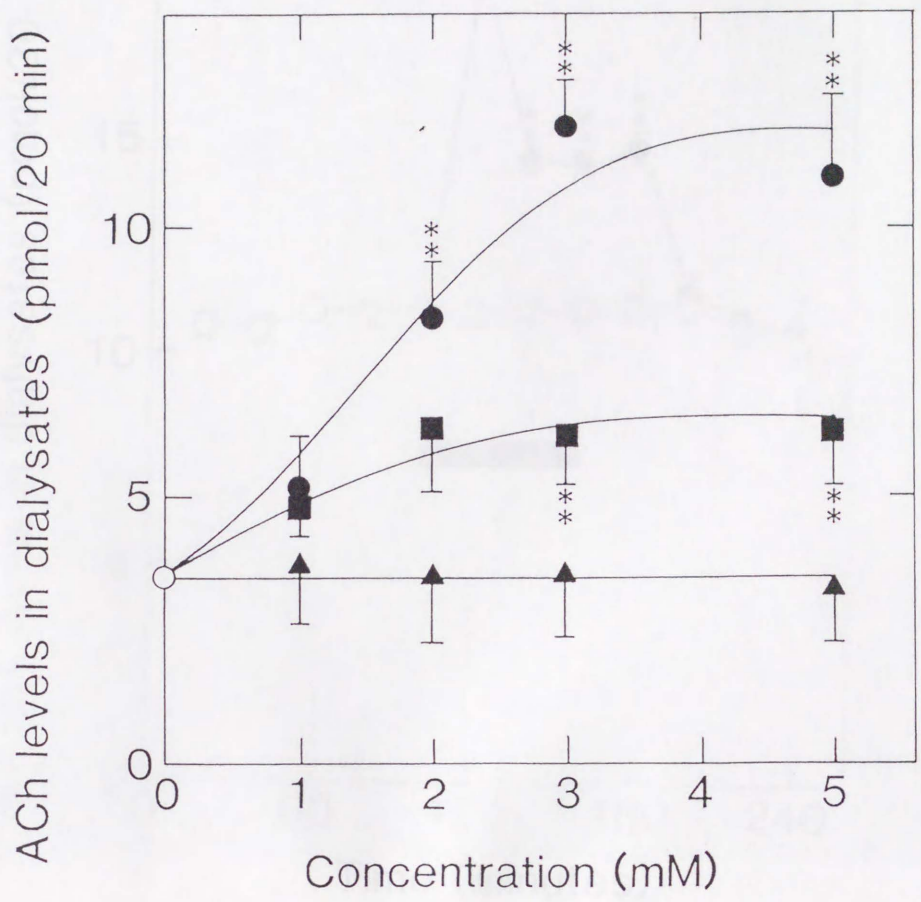


Table II

Effects of administration of L-DOPA or 5-HTP on monoamine content in the hippocampus

Each value is the mean ± S.E.M. of 4 determinations.

*, $p < 0.05$; **, $p < 0.01$; significantly different from the corresponding control values.

	Noradrenaline (nmol/g tissue)	Dopamine (nmol/g tissue)	5-Hydroxytryptamine (nmol/g tissue)
vehicle	3.06 ± 0.17	0.185 ± 0.008	1.26 ± 0.16
L-DOPA, 50mg/kg	3.37 ± 0.29	0.200 ± 0.013	1.28 ± 0.17
L-DOPA, 100 mg/kg	3.62 ± 0.21*	0.330 ± 0.029**	1.29 ± 0.27
L-DOPA, 200 mg/kg	4.09 ± 0.35**	0.528 ± 0.036**	1.27 ± 0.25
5-HTP, 50 mg/kg	3.11 ± 0.13	0.184 ± 0.012	2.43 ± 0.21**
5-HTP, 100 mg/kg	3.00 ± 0.28	0.177 ± 0.020	3.58 ± 0.37**
5-HTP, 200 mg/kg	3.18 ± 0.18	0.180 ± 0.019	6.82 ± 1.43**

Fig. 3

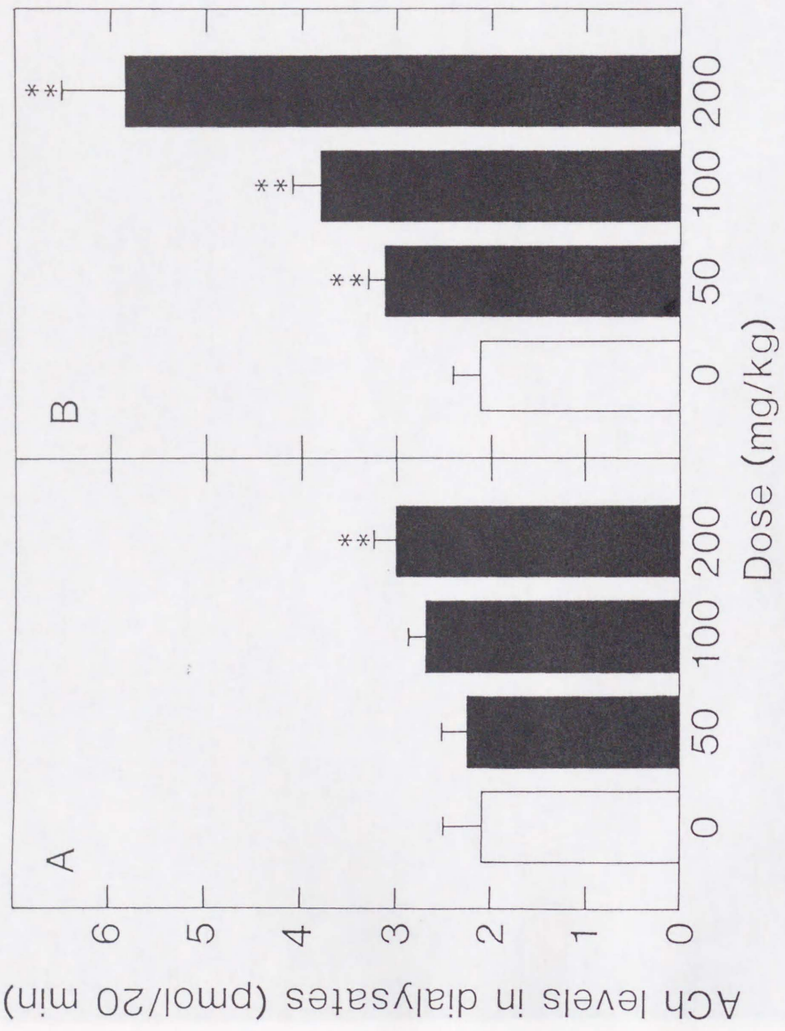
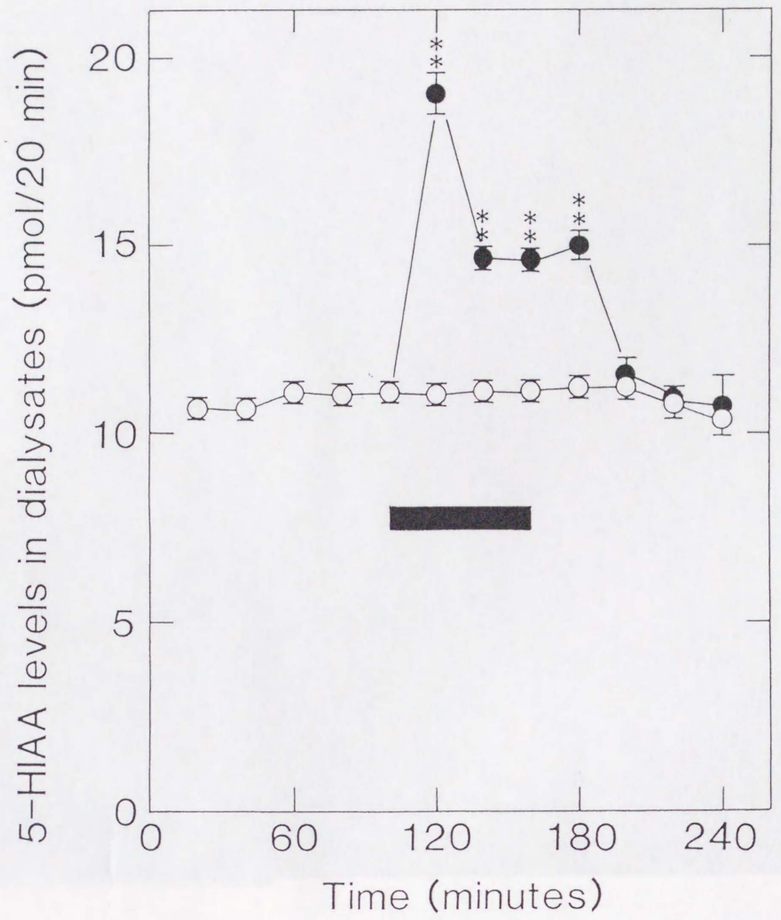

A
B
**
0
50
100
200
Dose (mg/kg)
0
1
2
3
4
5
6
ACh levels in dialysates (pmol/20 min)

Fig. 4


**
0
5
10
15
20
0
60
120
180
240
Time (minutes)
5-HIAA levels in dialysates (pmol/20 min)